Application Note

Performance of Estapor® Microspheres and Hi-Flow™ Plus Membranes in a Lateral Flow Assay for Human Chorionic Gonadotropin (hCG)

Summary

Estapor® carboxyl-modified dyed microspheres and Hi-Flow™ Plus lateral flow membranes were used in the assembly of lateral flow test strips for the detection of human chorionic gonadotropin (hCG). By pairing microspheres of diameters ranging from 0.185 to 0.478 µm with membranes of different flow rates, we show quantitatively that microspheres of 0.478 µm diameter produced the highest signals on all but the slowest membrane, Hi-Flow™ Plus 180. Microspheres with a diameter of 0.185 µm produced the lowest signals at all hCG concentrations. Microspheres with diameters of 0.228, 0.413, and 0.422 µm produced intermediate signal intensities that were similar on each type of membrane. False positives with negative controls were observed on test strips run with 0.478-µm microspheres. These nonspecific signals were eliminated by optimization of the conjugation protocol.

Introduction

Lateral flow test strips are immunochromatographic assays used to test for an array of target analytes. Creating a functional and sensitive lateral flow assay draws on principles from biology, chemistry, physics and engineering. These are explored in Rapid Lateral Flow Test Strips: Considerations for Product Development (Lit No. TB500EN00EM, Rev C, EMD Millipore, 2013).

While several types of detector particles can be used in the manufacture of lateral flow assays, latex microspheres are the most versatile of the particles currently used. In addition to being available in a wide range of sizes, they can be detected by colorimetry, fluorometry, or paramagnetism, depending on the specific particle composition. They are also available with a range of coupling chemistries for covalent attachment of antibodies and other types of molecules.

In lateral flow assays, the detector particles are required to move through the porous structure of the membrane. The size of the particles will affect their mobility due to physical interactions with the pore walls. Thus, small particles will move faster than larger particles. Assuming that the particle population is monodisperse with uniform size and shape, differences in assay sensitivity and specificity may be expected. The inherent flow rate of the membrane also affects the rate of particle movement. The net result is that an assay manufacturer must determine the appropriate microsphere size and membrane flow rate to achieve the desired levels of sensitivity and specificity.

In this report, we examine the relationship between microsphere size, membrane flow rate, and assay sensitivity, using blue Estapor® carboxyl-modified dyed microspheres and Hi-Flow™ Plus membranes.

EMD Millipore is a division of Merck KGaA, Darmstadt, Germany

Methods

Lateral flow test strips for the detection of hCG were assembled using blue Estapor® carboxyl–modified dyed microspheres (Table 1), Hi-Flow™ Plus lateral flow membranes (Table 2) and anti-hCG antibodies (Fitzgerald Industries (Table 3)).

Microspheres were conjugated to test antibodies using a two-step EDC/Sulfo-NHS covalent coupling procedure. Microspheres were washed twice with 50 mM MES buffer (pH 6.0) before activation with EDC and Sulfo-NHS. Following activation, the microspheres were washed with 50 mM MES buffer (pH 6.0), coated with 60 mg beta hCG antibody #10-C25E (Fitzgerald) per gram of microspheres, and mixed on a rotary wheel for 2 hours at room temperature. (60 mg of antibody was found to be optimal in previous experiments.) Following antibody coating, the microspheres were quenched with 30 µL of ethanolamine and mixed on a rotary wheel at room temperature for 30 minutes. The microspheres were washed twice and blocked in 50 mM Tris (pH. 8.0), 0.5% casein for 2 hours by mixing at room temperature on a rotary wheel. Finally, the microspheres were resuspended to a final concentration of 1% (w/v) in 50 mM Tris (pH. 8.0), 0.5% casein. For conjugate pad application, the hCG conjugates were diluted to 0.065% (w/v) in 50 mM Tris (pH 8.0) containing 0.5% casein, 2.5% trehalose, 10% sucrose, and 0.5% polyvinylpyrrolidone. An aliquot of 1 mL microsphere suspension was applied to each glass fiber conjugate pad (0.5 x 30 cm, SureWick® G041 Glass Fiber Pad, EMD Millipore) to yield about 10.8 µg of microspheres per test. Conjugate pads were dried overnight at room temperature and stored in a sealed foil pouch with desiccant sachets until used for test strip assembly.

Hi-Flow™ Plus membranes were striped with capture reagent solutions using a Kinematic Matrix 1600 Reagent Dispensing Module under these conditions:

Relative humidity: 40% to 60%
Temperature: 18–22 °C
Bed speed: 10 cm/sec
Reagent dispense rate: 10 µL/sec

The test line solution contained hCG beta antibody #10-C25D (Fitzgerald) at a concentration of 1 mg/mL in 50 mM MES (pH 6.0). The control line solution contained a polyclonal goat IgG, raised against mouse IgG, at a concentration of 1 mg/mL in Milli-Q® water. Membranes were dried at 37 °C for 2 hours and then assembled together with conjugate pads, sample pads (SureWick® C083 Cellulose Fiber Pad treated with 10mM Tris (pH 8.2), 1% Tween 20, 0.75% BSA) and absorbent pads (SureWick® C083 Cellulose Fiber Pad, EMD Millipore) into lateral flow test strips (Figure 1).

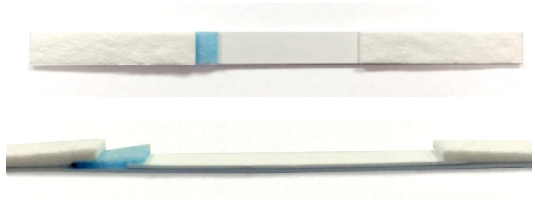

Figure 1. Schematic view of a lateral flow test strip

The functionality and sensitivity of the lateral flow test strips were evaluated with a range of hCG (BBI Solutions) concentrations prepared in human urine (Biochemical Diagnostics Inc). Microsphere mobility and nonspecific binding were evaluated visually. Signal intensity at the test lines was evaluated visually and also quantitatively using an ESEQuant Lateral Flow Immunoassay Reader (Qiagen). Photographs of selected test strip series have been included where appropriate.

Catalogue No.	Diameter (µm)	µeq COOH/g
K1-010	0.185	516
K1-030	0.288	367
K1-030	0.413	284
R06-22	0.422	21
K1-050	0.478	8

Table 1. Estapor® Carboxyl-Modified Dyed Microspheres

Catalogue No.	Membrane Type
SHF0750225	Hi-Flow™ Plus 75
SHF0900425	Hi-Flow™ Plus 90
SHF1200425	Hi-Flow™ Plus 120
SHF1350425	Hi-Flow™ Plus 135
SHF1800425	Hi-Flow™ Plus 180

Table 2. Hi-Flow™ Plus Membranes

Catalogue No.	Antibody
10-C25D	hCG beta antibody (capture)
10-C25E	hCG beta antibody (detector)

Table 3. Fitzgerald hCG Antibodies

Results

After constructing and running test strips with hCG samples of known concentration, the signal intensities at the test lines were evaluated colorimetrically. For each combination of microsphere size and membrane speed, the data were graphed and are presented below.

On Hi-Flow™ Plus 75 (Figure 2a) and Hi-Flow™ Plus 90 (Figure 2b), the fastest flowing membranes, the highest signal intensities from the quantitative analysis were obtained with the 0.478-µm diameter microspheres; and the lowest signal intensities were obtained with the 0.185-µm diameter microspheres. Interestingly, the microspheres of intermediate diameters (0.288, 0.413, and 0.422 µm) produced similar signal intensities.

Test strips run with the 0.478-µm microspheres exhibited two artifacts. First, a quantifiable signal was detected on the negative control strip (i.e., 0 mIU hCG/mL) on Hi-Flow™ Plus 90. Second, faint lines were visible above and below the primary control line (Figure 2c). These represent the edges of the liquid line when the control line reagent was initially applied to the membrane. Another feature that can be seen is a decrease in the intensity of the control line as the intensity of the test line increases. This is expected, given that the control line is capturing only the microspheres that travel beyond the test line.

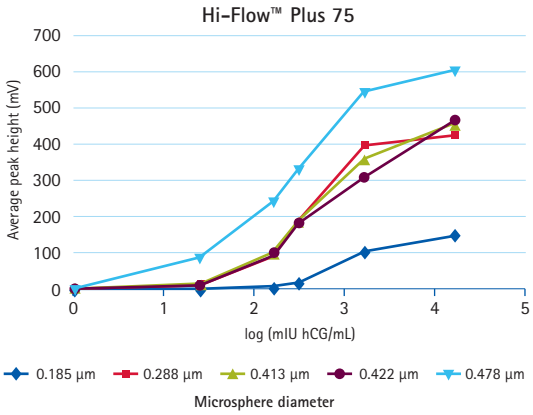

Figure 2a. Signal intensity for hCG detection on Hi-Flow™ Plus 75 using Estapor® microspheres of different diameters.

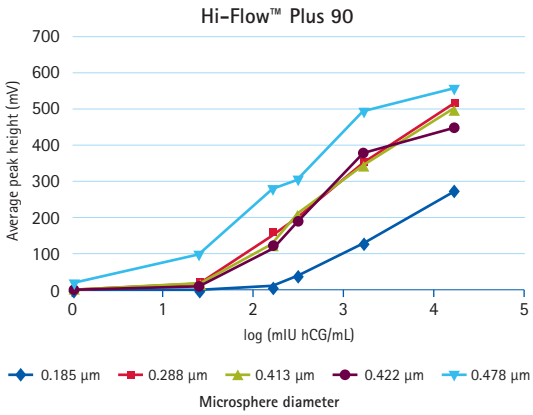

Figure 2b. Signal intensity for hCG detection on Hi-Flow™ Plus 90 using Estapor® microspheres of different diameters.

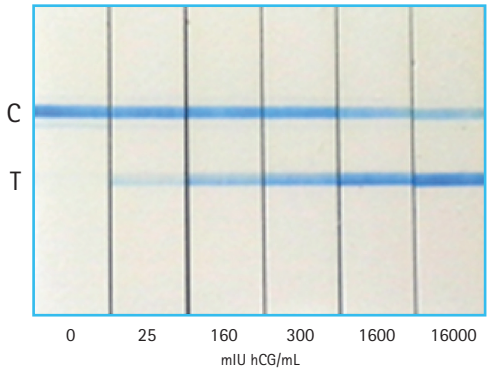

Figure 2c. Sensitivity of hCG detection on Hi-Flow™ Plus 75. Estapor® microspheres with a diameter of 0.478 µm were used (C = control line; T = test line).

When the signal intensities were evaluated on Hi-Flow™ Plus 120 (Figure 3a) and Hi-Flow™ Plus 135 (Fig 3b), similar trends were observed. The highest signal intensities were obtained with the 0.478-µm diameter microspheres; and the lowest signal intensities were obtained with the 0.185-µm diameter microspheres. The microspheres of intermediate diameters (0.288, 0.413, and 0.422 µm) also produced similar signal intensities. As with Hi-Flow™ Plus 90, a quantifiable signal was detected in the negative control with the 0.478-µm microspheres (Figure 3c, 3d). Smaller microspheres did not produce a nonspecific signal on the negative control strips (Figure 3c, 3d).

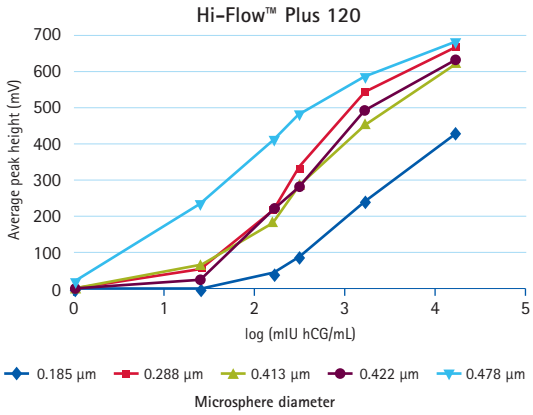

Figure 3a. Signal intensity for hCG detection on Hi-Flow™ Plus 120 using Estapor® microspheres of different diameters.

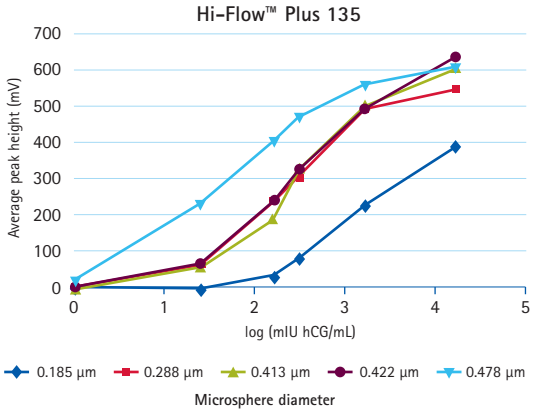

Figure 3b. Signal intensity for hCG detection on Hi-Flow™ Plus 135 using Estapor® microspheres of different diameters.

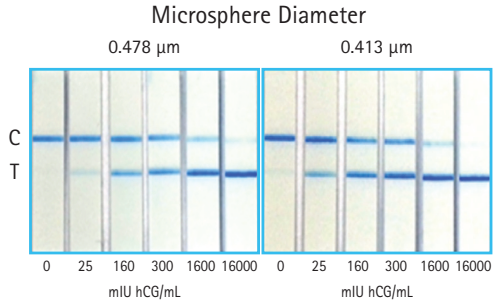

Figure 3c. Sensitivity of hCG detection on Hi-Flow™ Plus 120. Estapor® microspheres with a diameter of 0.478 µm and 0.413 µm were used (C = control line; T = test line).

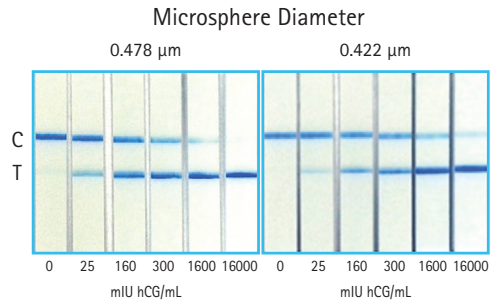

Figure 3d. Sensitivity of hCG detection on Hi-Flow™ Plus 135. Estapor® microspheres with a diameter of 0.478 µm and 0.422 µm were used (C = control line; T = test line).

On Hi-Flow™ Plus 180, the slowest flowing membrane, the trends were generally the same (Figure 4a). The highest signal intensities were obtained with the 0.478-µm diameter microspheres; and the lowest signal intensities were obtained with the 0.185-µm diameter microspheres. The microspheres of intermediate diameters (0.288, 0.413, and 0.422 µm) produced similar signal intensities. One significant difference occurred with 0.478-µm microspheres. Above 160 mIU hCG/mL, the signal intensity levelled off well below the values observed on the faster flowing membranes. A quantifiable signal was also measured on the negative control.

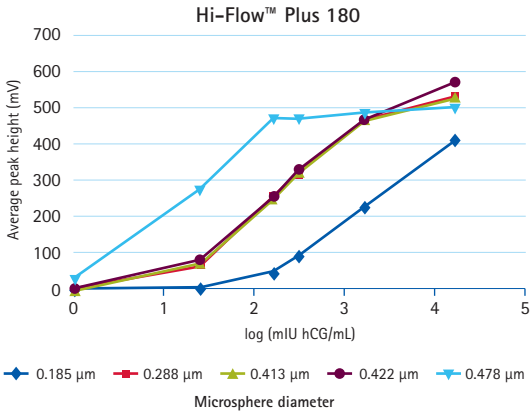

Figure 4a. Signal intensity for hCG detection on Hi-Flow™ Plus 180 using Estapor® microspheres of different diameters.

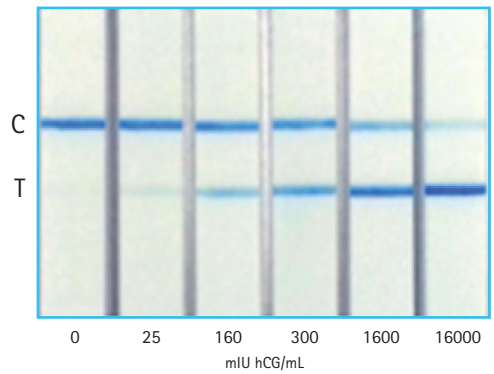

Figure 4b. Sensitivity of hCG detection on Hi-Flow™ Plus 180. Estapor® microspheres with a diameter of 0.288 µm were used (C = control line; T = test line).

On all Hi-Flow™ Plus membranes, the results obtained with the 0.413-µm and 0.422-µm Estapor® microspheres indicate that the difference in COOH charge density (21 vs 284 µEq/g) did not affect their performance.

Optimization of 0.478-µm Estapor® Microspheres

The measurable signals observed with the 0.478-µm microspheres on the hCG-negative controls indicated that nonspecific binding was occurring at the test line. Attempts to eliminate nonspecific binding by reducing the amount of microspheres in the assay were not fully successful. Thus, we investigated modifications to the conjugation procedure. The most effective approaches were optimizing the quenching and blocking steps. Specifically, the amount of ethanolamine used to quench the microspheres was reduced by 50% and the blocking agent was changed from 0.5% (w/v) casein to 1% (w/v) fish skin gelatin.

The optimized 0.478-µm microspheres were re-evaluated in the hCG assay on Hi-Flow™ Plus 75, Hi-Flow™ Plus 135, and Hi-Flow™ Plus 180 membranes. On Hi-Flow™ Plus 75, the signal intensity was reduced at all concentrations compared to the standard microspheres (Figure 5a). On the control lines, the faint lines above and below the primary line, which were observed with standard microspheres (Figure 2c), were no longer present (Figure 5b).

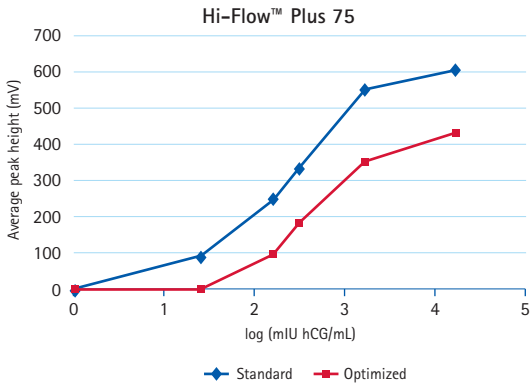

Figure 5a. Signal intensity for hCG detection on Hi-Flow™ Plus 75 using standard and optimized 0.478-µm Estapor® microspheres.

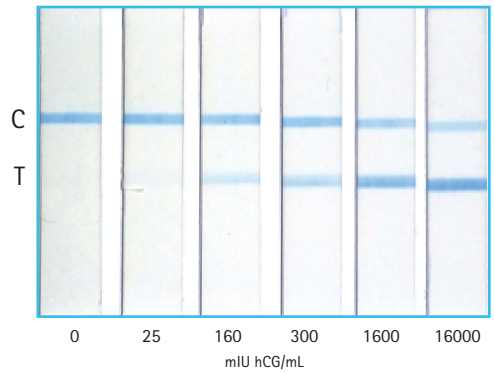

Figure 5b. Sensitivity of hCG detection on Hi-Flow™ Plus 75 using optimized 0.478-µm Estapor® microspheres. (C = control line; T = test line)

On Hi-Flow™ Plus 135 and Hi-Flow™ Plus 180, reduced signal intensities were observed at concentrations lower than 1600 mIU hCG/mL (Figures 6a and 7a). At 1600 mIU hCG/mL and above, the optimized and standard microspheres produced similar signal intensities. When the signal intensity profile on Hi-Flow™ Plus 135 was compared to the results obtained with smaller microspheres, it was found to be essentially the same (Figure 6b). Optimization of the 0.478-µm microspheres resulted in a reduction of specific signal intensity so that there appears to be no performance advantage relative to the smaller microspheres.

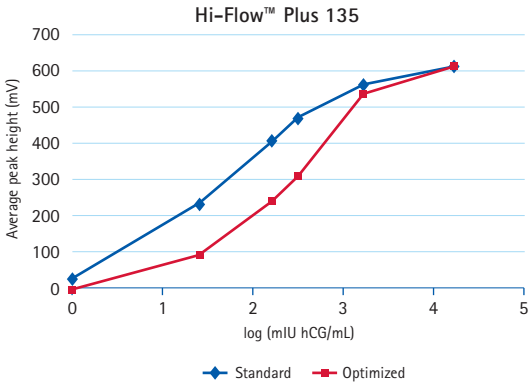

Figure 6a. Signal intensity for hCG detection on Hi-Flow™ Plus 135 using standard and optimized 0.478-µm Estapor® microspheres.

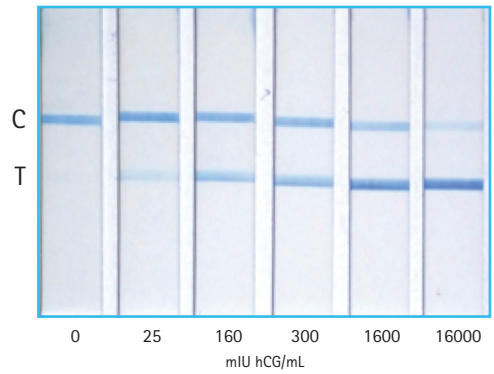

Figure 6b. Sensitivity of hCG detection on Hi-Flow™ Plus 135 using optimized 0.478-µm Estapor® microspheres. (C = control line; T = test line)

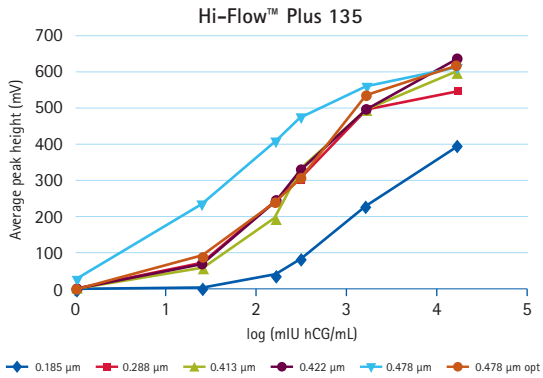

Figure 6c. Sensitivity of optimized 0.478-µm Estapor® microspheres compared to microspheres of other sizes.

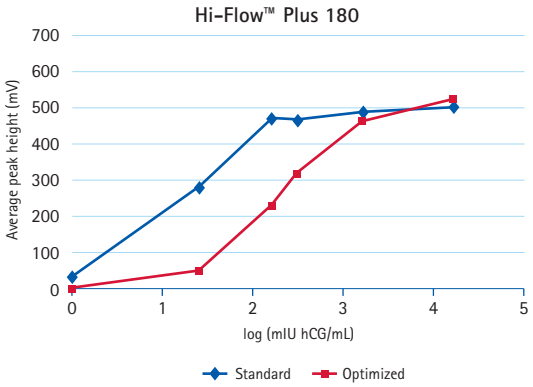

Figure 7a. Signal intensity for hCG detection on Hi-Flow™ Plus 180 using standard and optimized 0.478-µm Estapor® microspheres.

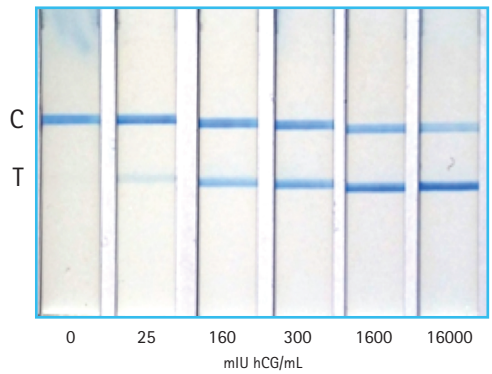

Figure 7b. Sensitivity of hCG detection on Hi-Flow™ Plus 180 using optimized 0.478-µm Estapor® microspheres. (C = control line; T = test line)

CONCLUSION

In this series of experiments using hCG detection as a model system, we have demonstrated the efficacy of Estapor® microspheres in lateral flow test strips manufactured on Hi-Flow™ Plus membranes. The optimal microsphere diameter for any given membrane has to take into account the sensitivity and specificity required in the final test strip design. Artifacts also need to be minimized so that interpretation of results is unambiguous.

Estapor® microspheres of 0.185 µm diameter were found to be the poorest performing regardless of membrane flow rate. Signals were undetectable at 25 mIU hCG/mL at all flow rates, and signal intensity at the control lines was uniformly less intense compared to larger microspheres. It is believed that these particles are flowing too rapidly to be efficiently captured at the test and control lines and that the color density of the particles is too low for them to be detected at the same efficiency as larger microspheres.

For all but Hi-Flow™ Plus 180, Estapor® microspheres with a diameter of 0.478 µm produced the highest signals. While this suggests that these microspheres are the best choice for Hi-Flow™ Plus 75, 90, 120, and 135, the artifacts that accompanied these higher signals require consideration of other aspects of the performance of the test strips. Optimization of conjugation to the 0.478-µm microspheres eliminated the nonspecific signals at 0 mIU hCG/mL observed on all membranes but also reduced the signal intensity for hCG-positive samples. Thus, there appears to be no performance advantage.

For Hi-Flow™ Plus 180, Estapor® microspheres from 0.288 to 0.422 µm diameter were found to give comparable performance up to a concentration of 16,000 mIU hCG/mL. Signal intensity did not level off with increasing hCG concentration as was observed with the 0.478-µm diameter microspheres. Thus, these microspheres appear to be interchangeable.

In conclusion, Estapor® microspheres ranging from 0.288 to 0.478 µm diameter were found to be effective as detector particles in lateral flow assays configured on Hi-Flow™ Plus membranes. While we have provided general guidelines on pairing microspheres with the membranes, test strip manufacturers should optimize each combination within the specifications for sensitivity and specificity that are required for the assay to be acceptable in the marketplace.

Additional Reading

a. Rapid Lateral Flow Test Strips: Considerations for Product Development (Lit No. TB500EN00EM, Rev C, EMD Millipore, 2013).

b. Estapor® Dyed Microspheres: A critical raw material for the manufacture of IVD and life sciences reagents (Lit No. PB4266EN00EM DI-12-06851, EMD Millipore, 2012).

c. Puertas S., et al., Designing novel nano-immunoassays: antibody orientation versus sensitivity. J. Phys. D: Appl. Phys. (2010) 43:474012 (8 pp.).

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